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(54) Title: METHODS FOR ENHANCING THE DISEASE RESISTANCE OF PLANTS

(57) Abstract

The invention relates to the genetic manipulation of plants to enhance disease resistance. The methods involve genetically manipulating plants to produce a polyketide that induces the accumulation of defense-related proteins in a plant. Such methods find use in agriculture, particularly in lessening the impact of disease-causing organisms on crop plants. Methods for genetically manipulating plats to produce such a polyketide are provided. Transformed plants, plant cells, plant tissues and seeds thereof are also provided.

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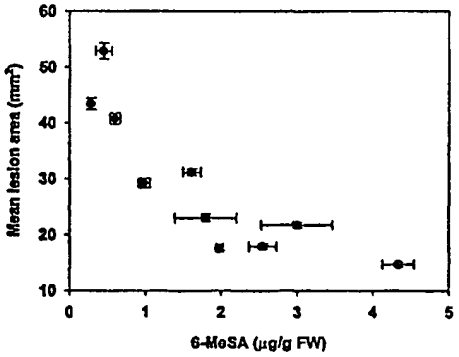

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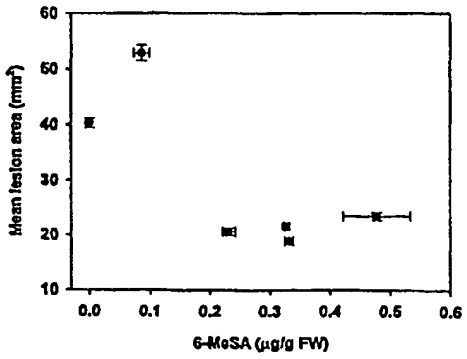

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METHODS FOR ENHANCING THE DISEASE RESISTANCE OF PLANTS

FIELD OF THE INVENTION

The invention relates to the genetic manipulation of plants, particularly to transforming plants with genes that enhance disease resistance.

BACKGROUND OF THE INVENTION

Disease in plants is caused by biotic and abiotic causes. Biotic causes include fungi, viruses, bacteria, and nematodes. Of these, fungi are the most frequent causative agent of disease on plants. Abiotic causes of disease in plants include extremes of temperature, water, oxygen, soil pH, plus nutrient-element deficiencies and imbalances, excess heavy metals, and air pollution.

A host of cellular processes enables plants to defend themselves from disease caused by pathogenic agents. Subsequent to recognition of a potentially pathogenic microbe, plants can activate an array of biochemical responses. Generally, the plant responds by inducing several local responses in the cells immediately surrounding the infection site. The most common resistance response observed in both nonhost and race-specific interactions is termed the "hypersensitive response" (HR). In the hypersensitive response, cells contacted by the pathogen, and often neighboring cells, rapidly collapse and dry in a necrotic fleck. Other responses include the deposition of callose, the physical thickening of cell walls by lignification, and the synthesis of various antibiotic small molecules and proteins. Genetic factors in both the host and the pathogen determine the specificity of these local responses, which can be very effective in limiting the spread of infection.

In addition to the localized hypersensitive response, plants have evolved a systemic defense system that reduces the impact of subsequent pathogen attacks. In 1961, Ross (*Virology 14*: 340-358) reported that infections of tobacco mosaic virus on tobacco were restricted by a prior infection with tobacco mosaic virus. Ross also reported that this induced resistance was also effective against tobacco necrosis virus and certain bacterial pathogens. Furthermore, the induced resistance

was both local and systemic in that both the originally infected leaves as well as leaves that were not previously infected with tobacco mosaic virus displayed increased resistance to pathogens. Ross coined the term "systemic acquired resistance" to refer to the inducible systemic resistance.

During the 1980's, systemic acquired resistance was studied intensively. The accumulation of a group of extracellular proteins called pathogenesis-related (PR) proteins were reported to correlate with the onset of SAR (Van Loon *et al.* (1982) *Neth. J. Plant. Path. 88*:237-256). Salicylic acid, a plant produced phenolic compound, was implicated as a signal in systemic acquired resistance based on the discovery that applying salicylic acid to plants induces both systemic acquired resistance and the accumulation of PR proteins (White, R. F. (1979) *Virology 99*:410-412. Despite two decades of intensive investigation, the exact role of salicylic acid in systemic acquired resistance remains unclear.

As noted, among the causative agents of infectious disease of crop plants, the phytopathogenic fungi play the dominant role. Phytopathogenic fungi cause devastating epidemics, as well as causing significant annual crop yield losses. All of the approximately 300,000 species of flowering plants are attacked by pathogenic fungi. However, a single plant species can be host to only a few fungal species and similarly, most fungi usually have a limited host range.

Plant disease outbreaks have resulted in catastrophic crop failures that have triggered famines and caused major social change. Generally, one of the best strategies for plant disease control is to use resistant cultivars selected or developed by plant breeders for this purpose. However, the potential for serious crop disease epidemics persists today, as evidenced by outbreaks of the Victoria blight of oats and southern corn leaf blight. Accordingly, molecular methods are needed to supplement traditional breeding methods to protect plants from pathogen attack.

SUMMARY OF THE INVENTION

Methods are provided for enhancing the resistance of plants to pathogens. The methods of the invention find use in agriculture for controlling plant pathogens including fungi, bacteria, viruses and nematodes. Such methods involve increasing the level of at least one defense-related protein in a plant. The methods comprise stably transforming plants with a gene encoding a polyketide synthase

operably linked to a promoter that drives expression in a plant. Preferably, the gene encodes a type I polyketide synthase. More preferably, the gene encodes 6-methylsalicylic acid synthase.

Methods are provided comprising stably transforming a plant with a polyketide synthase gene and at least one additional gene. It is recognized that, in certain plants, or in certain compartments within a plant cell, there may be an insufficient level of a substrate or other key component involved in the synthesis of a polyketide *via* a polyketide synthase. It is also recognized that such an insufficiency may be overcome by stably transforming a plant with one or more additional genes. Such genes encode proteins that, for example, increase the level of a substrate for a polyketide synthase or convert the polyketide synthase from an inactive to active form. In a preferred embodiment of the invention, a plant is capable of expressing a gene encoding a 6-methylsalicylic acid synthase and a gene encoding a phosphopantetheinyl transferase.

Also provided are transformed plants, plant tissues, plant cells and seeds thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the results of a western blotting experiment comparing the levels of PR1-like proteins in B73 maize leaves seven days after infiltration with solutions containing either 6-methylsalicylic acid or salicylic acid.

Figure 2 depicts the results of a western blotting experiment comparing the levels of three PR proteins, PR1, glucanase and chitinase, in Xanthi-nc tobacco leaves seven days after infiltration with a solution containing either 2.5 mM 6-methylsalicylic acid or 2.5 mM salicylic acid.

Figure 3 is graphical representation of the results of an experiment comparing the antifungal activity of 6-methylsalicylic acid to structurally related compounds.

Figure 4 is a graphical representation of the relationship between the levels of PR1 and chitinase proteins and the level of 6-methylsalicylic acid in leaves of T_1 tobacco plants transformed with a 6-methylsalicylic acid synthase gene from *Penicillium patulum*.

Figure 5 is a graphical representation of the effect of the level of 6-methylsalicylic acid in leaf tissue on the resistance against tobacco mosaic virus in transgenic tobacco expressing a 6-methylsalicylic acid synthase gene from *Penicillium patulum*. Mean lesion diameter on leaves was measured four days after inoculation with TMV. (A) T_1 progeny of selfed T_0 plant SID #911403. (B) T_1 progeny of selfed T_0 plant SID #870955.

Figure 6 is a graphical representation of the effects of maturation on 6-methylsalicylic acid levels in the leaves of T_0 GS3 maize plants transformed with a 6-methylsalicylic acid synthase gene from *Penicillium patulum*. Leaf 6 of V8-stage maize was used for the first sampling. For the second sampling leaf 8 of plants that had formed ears were used.

Figure 7 is a graphical representation of the levels of 6-methylsalicylic acid and salicylic acid in maize kernels from T_0 GS3 maize plants that were transformed with a 6-methylsalicylic acid synthase gene from *Penicillium patulum*. The transformed GS3 plants were pollinated with HG11. Control kernels were from nontransgenic GS3 plants that were pollinated with HG11. The error bars represent standard error.

Figure 8 is a graphical representation of 6-methylsalicylic acid levels in leaves from T_1 maize plants that are progeny of T_0 GS3 plants transformed with a 6-methylsalicylic acid synthase gene from *Penicillium patulum*. To produce the T_1 plants, transformed T_0 GS3 plants were pollinated with HG11. Each data point represents the mean of two extracts from leaf 9 of each T_1 plant. The error bars represent standard error of the mean. Multiple V10-stage T_1 progeny from each T_0 GS3 parent were analyzed. Only T_1 plants that were determined to express the 6-methysalicylic acid synthase gene by RT-PCR were analyzed for 6-methylsalicylic acid accumulation. The SID# refers to the identity of the T_0 GS3 parent.

DETAILED DESCRIPTION OF THE INVENTION

Methods are provided for enhancing the resistance of plants to pathogens. The methods of the invention find use in preventing or reducing pathogen-inflicted damage to plants, particularly agricultural plants. The methods involve genetically manipulating a plant to alter the expression of its endogenous defense responses resulting in the accumulation of at least one defense-related protein in the plant.

Such methods provide plants and plant cells with enhanced resistance to pathogens.

By "resistance" is intended that the plants avoid or limit the disease symptoms which are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen is minimized or lessened. The methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

By "endogenous defense responses" is intended to include at least one of the components of one of the endogenous systems a plant can employ to limit or prevent the diseases and/or damage caused by pathogens. Such endogenous systems include, but are not limited to, hypersensitive responses, local and systemic acquired resistance, and wound responses Such systems encompass a variety of physiological processes including, for example, synthesis of pathogenesis-related proteins, programmed cell death, synthesis of phytoalexins, lignification of cell walls, formation of callose, synthesis of free radicals, synthesis of antibiotic molecules including proteins, evolution of antipathogenic volatiles, evolution of ethylene, and the like.

By "defense-related protein" is intended any protein produced by a plant that aids, either directly or indirectly, in defending a plant against pathogens and/or is altered in level or activity as a result of a pathogen impacting a plant. Such "defense-related proteins" include, but are not limited to, pathogenesis-related proteins, proteinase inhibitors, systemin, antibiotic proteins, and the like.

The methods of the invention provide plants and plant cells that are genetically manipulated to produce a polyketide that induces a plant's endogenous defense responses to pathogens. While the polyketide may be a functional analog of a naturally occurring inducer of defense responses such as salicylic acid, the invention encompasses any polyketide that induces a plant's endogenous defense responses. Such a polyketide is capable of inducing the accumulation of a defense-related protein in a plant. While the invention is not dependent on a specific polyketide, a preferred polyketide is 6-methylsalicylic acid.

The methods of the invention comprise stably incorporating in the genome of a plant a DNA construct comprising a nucleotide sequence encoding a

polyketide synthase operably linked to a promoter that drives expression in a plant. The polyketide synthase utilized is one that catalyzes the formation of a polyketide which is capable of inducing the accumulation of a defense-related protein in a plant. Preferably, such a polyketide synthase is a type I polyketide synthase. More preferably, the polyketide synthase is 6-methylsalicylic acid synthase. Most preferably, the polyketide synthase is a 6-methylsalicylic acid synthase encoded by a nucleotide sequence selected from the group consisting of EMBL Accession No. X55776, GenBank Accession No. U31329 and DDBJ Accession No. D85860 (SEQ ID NOs: 1-3, respectively).

The polyketide synthases of the invention include 6-methylsalicylic acid synthases. Such enzymes catalyze the formation of 6-methylsalicylic acid, a structural and functional analogue of salicylic acid, an endogenous, plant-defense-response signaling molecule. While the exact role of salicylic acid in plant defense responses is unclear, salicylic acid is known to induce the expression of genes encoding defense-related proteins in plants. This salicylic acid-induced increase in gene expression leads to the accumulation of defense-related proteins along with an increase in disease resistance. While the invention is not bound by any particular mechanism of action, the application of 6-methylsalicylic acid to plants also induces the accumulation of defense-related proteins and enhances disease resistance. Furthermore, plants transformed to express 6-methylsalicylic acid synthase produce 6-methylsalicylic acid and accumulate enhanced levels of defense proteins.

To achieve the desired level of a polyketide in a plant, a plant can be transformed with one or more nucleotide sequences encoding a protein that directly or indirectly affects the function of the polyketide synthases of the invention. Examples of a protein that indirectly affects the function of a polyketide synthase, include but are not limited to, an enzyme that catalyzes the synthesis of a substrate of a polyketide synthase and an enzyme which catalyzes the degradation or further metabolism of the polyketide. An example of such an enzyme is UDP-glucose:salicylic acid glucosyltransferase. This enzyme catalyzes the transfer of a glucosyl residue from UDP-glucose to salicylic acid. Recently, a tobacco UDP-glucose:salicylic acid glucosyltransferase was purified and its corresponding cDNA was cloned and sequenced (Lee and Raskin (1999) *J. Biol. Chem.*

274:36637-36642). An example of a protein that directly affects the function of a polyketide synthase is the enzyme, phosphopantetheinyl transferase. This enzyme converts a polyketide synthase from an inactive form into an active form by catalyzing one or more transfers of the phosphopantetheinyl moiety of Coenzyme A to a serine residue in the acyl carrier domain of the polyketide synthase.

The methods of the invention comprise stably incorporating in the genome of a plant at least one additional DNA construct comprising a nucleotide sequence encoding a protein that affects the function of a polyketide synthase, operably linked to a promoter that drives expression in a plant. Depending on the desired outcome, a plant can be transformed with such a nucleotide sequence in either the sense orientation or antisense orientation. Generally, the sense orientation is utilized to increase the level of a protein in a plant, and the antisense orientation is utilized to decrease the level of a protein in the plant. In a preferred embodiment of the invention, the additional DNA construct comprises a nucleotide sequence encoding a phosphopantetheinyl transferase in the sense orientation operably linked to a promoter. Preferably, the nucleotide sequence encoding the phosphopantetheinyl transferase is selected from the group consisting of EMBL Accession No. A36232, GenBank Accession No. L17438, EMBL Accession Nos. X65610 and X63158 and DDBJ Accession Nos. D50562 and D21876 (SEQ ID NOs: 4-9, respectively).

It is recognized that one or more enzymes may be present in a plant that use the polyketide of the invention as a substrate. It is also recognized that the presence of such an enzyme in a plant may be undesirable because its associated enzymatic activity can decrease the level of the desired polyketide in a plant. In a preferred embodiment of the invention, the additional DNA construct comprises a nucleotide sequence encoding a glucosyltransferase in the antisense orientation operably linked to a promoter that drives expression in a plant cell. The glucosyltransferase selected is capable of catalyzing the synthesis of 6-methylsalicylic acid glucosides, including, but not limited to, the glucosyltransferases encoded by GenBank Accession Nos. U32643 and U32644 (SEQ ID NOs: 10-11, respectively) and the tobacco UDP-glucose:salicylic acid glucosyltransferase encoded by the nucleotide sequence reported by Lee and Raskin ((1999) *J. Biol. Chem. 274*:36637-36642) (SEQ ID NO: 12). Antisense

expression of a nucleotide sequence of such a glucosyltransferase can eliminate or reduce the synthesis of 6-methylsalicylic acid glucosides in a plant, resulting in an increased level of 6-methylsalicylic acid in the plant.

The nucleotide sequences encoding the polyketide synthases and additional proteins of the invention are operably linked to promoters that drive expression of the sequence in a plant cell. Any one of a variety of promoters can be used with the sequences of the invention depending on the desired timing and location of expression. Preferred promoters include constitutive, pathogen-inducible, insect-inducible, nematode-inducible, wound-inducible, tissue-preferred, developmentally regulated and chemically regulatable promoters.

The polyketide synthases and additional proteins of the invention can be targeted to a specific cellular compartment. It is recognized that the substrates for the polyketide may occur in specific cellular compartments. Such compartments include, for example, the cytosol, chloroplast, mitochondrion and vacuole.

The invention encompasses plants transformed with the polyketide synthases of the invention and seeds thereof. The invention also encompasses transformed plant cells and plant tissues.

The invention provides plants transformed with nucleotide sequences encoding 6-methylsalicylic acid synthase. Such plants, or parts thereof, can produce substantial levels of 6-methylsalcylic acid. Plant parts of the invention are any part of a plant including, but not limited to, seeds, fruits, tubers, leaves, roots and flowers. Because 6-methylsalicylic acid is structurally similar to the known pharmacological agents, acetylsalicylic acid (aspirin) and salicylic acid, plant parts that accumulate 6-methylsalicylic acid may serve as therapeutic agents in therapeutic methods for humans and livestock. Such therapeutic methods involve administering a therapeutically effective amount of a plant part that contains 6-methylsalicylic acid. Alternatively, 6-methylsalicylic acid can be substantially purified from the plant or part thereof by methods known in the art, and such substantially purified 6-methylsalicylic acid can be administered. By "therapeutically effective amount" is intended an amount of a the plant part or product thereof, such as, for example, 6-methylsalicylic acid, that can alleviate or eliminate the effects of a malady when such a plant part or product is ingested or administered by any method known in the art. Any of the maladies that are known

in the art for which acetylsalicylic and other salicylates are used as therapeutic agents may be treated by the methods of the present invention.

The polyketide synthases and additional proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polyketide synthases can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel, T. (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra (eds.) *Techniques in Molecular Biology*, MacMillan Publishing Company, NY (1983) and the references cited therein. A mutagenic and recombinogenic procedure such as DNA shuffling may be employed to alter the polyketide synthases and other proteins of the invention (see U.S. Pat. Nos. 5,6057,93 and 5,837,458). Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired enzyme activity or other function. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

In this manner, the present invention encompasses the polyketide synthases as well as components and fragments thereof. That is, it is recognized that component polypeptides or fragments of the proteins may be produced which retain polyketide synthase activity. These fragments include truncated sequences, as well as N-terminal, C-terminal, internal and internally deleted amino acid sequences of the proteins.

The deletions, insertions, and substitutions of the protein sequence encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening

assays. That is, the enzyme activity of a polyketide synthase can be evaluated by standard methods known to those of ordinary skill in the art. See, for example, methods for measuring the activity of 6-methylsalicylic acid synthase (Spencer and Jordan, (1992) *Biochem. J. 288*:839-846). Alternatively, the accumulation of the polyketide product can be measured. See, for example, the methods disclosed in Example 4.

The polyketide synthases described herein may be used alone or in combination with additional proteins or agents to protect against plant diseases and pathogens.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. Constitutive, tissue-preferred pathogen-inducible and wound-inducible promoters may be used to control the expression of the genes encoding the polyketide synthases of the invention. If constitutive expression of the polyketide synthase is desired a constitutive promoter can employed. Constitutive promoters include for example, the 35S CaMV and U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142.

Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol Gen Genet. 254(3)*:337-343; Russell *et al.* (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505. Such promoters can be modified, if necessary, for weak expression.

Where low level expression is desired, weak promoters will be used. It is recognized that weak inducible promoters may be used. Additionally, either a weak constitutive or a weak tissue-preferred promoter may be used.

Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By low level is intended at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000

transcripts. Alternatively, it is recognized that weak promoters also encompass promoters that are expressed in only a few cells and not in others to give a total low level of expression. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Such weak constitutive promoters include, for example, the core promoter of the Rsyn7 (copending application serial number 08/661,601), the core 35S CaMV promoter, and the like.

If expression of the polyketide synthase is desired in response to a pathogen attack, a pathogen-inducible promoter can be utilized. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; *e.g.*, PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *The Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116.

When expression is desired in the vicinity of the infection site, promoters that are expressed locally at or near the site of pathogen infection may be used. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Molecular and General Genetics* 2:93-98; and Yang, Y (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang and Sing (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; Cordero *et al.* (1992) *Physiological and Molecular Plant Pathology* 41:189-200; and the references cited therein.

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound inducible promoter may be used in the constructions of the invention. Such wound inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan, C., *Ann. Rev Phytopath. 28*:425-449; Duan *et al. Nature Biotechnology 14*:494-498); wun1 and wun2, U.S. Patent No. 5,428,148; win1 and win2 (Stanford *et al. Mol Gen Genet 215*:200-208); systemin (McGurl *et al. Science 225*:1570-1573); WIP1 (Rohmeier *et al. Plant Mol Biol. 22*:783-792;

Eckelkamp *et al. FEBS Letters 323*:73-76); MPI gene (Corderok *et al. The Plant Journal 6(2)*:141-150); and the like, herein incorporated by reference.

The polyketide synthases and additional proteins of the invention can be targeted to a specific cellular compartment. Methods for targeting a protein to a specific cellular compartment are known to those of ordinary skill in the art. If the desired subcellular location of polyketide synthase of the invention is the chloroplast, a chloroplast-targeting sequence can be operably linked to the coding sequence of the polyketide synthase. Chloroplast-targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol. 30*:769-780; Schnell *et al.* (1991) *J. Biol. Chem. 266(5)*:3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb. 22(6)*:789-810); tryptophan synthase (Zhao *et al.* (1995) *J.Biol. Chem. 270(11)*:6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem. 272(33)*:20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem. 268(36)*:27477-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem. 263*:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun. 196*:1414-1421; and Shah *et al.* (1986) *Science 233*:478-481.

It is recognized that with the nucleotide sequences of the invention, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the nucleotide sequences encoding the proteins of the invention can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation (cosuppression) are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The genes encoding the polyketide synthases and additional proteins of the invention can be introduced into any plant. The genes to be introduced can be conveniently used in expression cassettes for introduction and expression in any plant of interest.

Such expression cassettes will comprise a transcriptional initiation region linked to the polyketide synthase sequence of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By foreign is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from

another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* 1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

The expression cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on another expression cassette. Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences, which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5'-leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5'-noncoding region) (Elroy-Stein *et al.*. (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling *et al.* (1987) *Nature 325*:622-625; tobacco mosaic virus leader

(TMV), (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiology 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.* transitions and transversions, may be involved.

The polyketide synthase genes of the present invention can be used to transform any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols may vary depending on the type of plant or plant cell, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) Biotechniques *4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see, Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile

Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooydaas-Van Slogteren *et al.* 1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) In *The Experimental Manipulation of Ovule Tissues* ed. G.P. Chapman *et al.*, pp. 197-209. Longman, NY (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou *et al.* (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells, which have been transformed, may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In the methods of the invention, plants genetically manipulated to enhance disease resistance are utilized. By "genetically manipulated" is intended modifying the genome of an organism, preferably a plant, including cells and tissue thereof, by any means known to those skilled in the art. Modifications to a genome include both losses and additions of genetic material as well as any sorts of rearrangements in the organization of the genome. Such modifications can be accomplished by, for example, transforming a plant's genome with a DNA construct containing nucleotide sequences which are native to the recipient plant, non-native or a combination of both, conducting a directed sexual mating or cross pollination within a single species or between related species, fusing or transferring nuclei, inducing mutagenesis and the like. In the practice of the present invention,

transformation and cross pollination are preferred means for genetically manipulating plants to enhance disease resistance.

In the practice of certain specific embodiments of the present invention, a plant is genetically manipulated to produce more than one enzyme or protein involved in polyketide synthesis. Those of ordinary skill in the art realize that this can be accomplished in any one of a number of ways. For example, each of the respective coding sequences for such enzymes can be operably linked to a promoter and then joined together in a single continuous fragment of DNA comprising a multigenic expression cassette. Such a multigenic expression cassette can be used to transform a plant to produce the desired outcome. Alternatively, separate plants can be transformed with expression cassettes containing one or a subset of the desired set of coding sequences. Transformed plants that express the desired activity can be selected by standard methods available in the art such as, for example, assaying enzyme activities, immunoblotting using antibodies which bind to the proteins of interest, assaying for the products of a reporter or marker gene, and the like. Then, all of the desired coding sequences can be brought together into a single plant through one or more rounds of cross pollination utilizing the previously selected transformed plants as parents.

Methods for cross pollinating plants are well known to those skilled in the art, and are generally accomplished by allowing the pollen of one plant, the pollen donor, to pollinate a flower of a second plant, the pollen recipient, and then allowing the fertilized eggs in the pollinated flower to mature into seeds. Progeny containing the entire complement of desired coding sequences of the two parental plants can be selected from all of the progeny by standard methods available in the art as described supra for selecting transformed plants. If necessary, the selected progeny can be used as either the pollen donor or pollen recipient in a subsequent cross pollination.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso

millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

The invention is drawn to compositions and methods for inducing resistance in a plant to plant pests. Accordingly, the compositions and methods are also useful in protecting plants against fungal pathogens, viruses, nematodes, insects and the like.

Pathogens of the invention include, but are not limited to, viruses or viroids, bacteria, insects, nematodes, fungi, and the like. Viruses include any plant virus, for example, tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc. Specific fungal and viral pathogens for the major crops include: Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae (Phomopsis sojae)*, *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium (Colletotichum truncatum)*, *Corynespora cassiicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, Soybean mosaic virus, *Glomerella glycines*, Tobacco Ring spot virus, Tobacco Streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, Tomato spotted wilt virus, *Heterodera glycines Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*,

Mycosphaerella brassiccola, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibater michiganese subsp. insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrichum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American Wheat Striate Virus, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Ustilago tritici*, *Tilletia indica*, *Rhizoctonia solani*, *Pythium arrhenomannes*, *Pythium gramicola*, *Pythium aphanidermatum*, High Plains Virus, European wheat striate virus; Sunflower: *Plasmophora halstedii*, *Sclerotinia sclerotiorum*, Aster Yellows, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Fusarium moniliforme* var. *subglutinans*, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae (Fusarium graminearum)*, *Stenocarpella maydi (Diplodia maydis)*, *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* O, T *(Cochliobolus heterostrophus)*,

Helminthosporium carbonum I, II & III *(Cochliobolus carbonum), Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum, Physoderma maydis, Phyllosticta maydis, Kabatiella maydis, Cercospora sorghi, Ustilago maydis, Puccinia sorghi, Puccinia polysora, Macrophomina phaseolina, Penicillium oxalicum, Nigrospora oryzae, Cladosporium herbarum, Curvularia lunata, Curvularia inaequalis, Curvularia pallescens, Clavibacter michiganense* subsp. *nebraskense, Trichoderma viride,* Maize Dwarf Mosaic Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, *Claviceps sorghi, Pseudonomas avenae, Erwinia chrysanthemi* pv. *zea, Erwinia carotovora, Corn stunt spiroplasma, Diplodia macrospora, Sclerophthora macrospora, Peronosclerospora sorghi, Peronosclerospora philippinensis, Peronosclerospora maydis, Peronosclerospora sacchari, Sphacelotheca reiliana, Physopella zeae, Cephalosporium maydis, Cephalosporium acremonium,* Maize Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus, Maize Stripe Virus, Maize Rough Dwarf Virus; Sorghum: *Exserohilum turcicum, Colletotrichum graminicola (Glomerella graminicola), Cercospora sorghi, Gloeocercospora sorghi, Ascochyta sorghina, Pseudomonas syringae* p.v. *syringae, Xanthomonas campestris* p.v. *holcicola, Pseudomonas andropogonis, Puccinia purpurea, Macrophomina phaseolina, Perconia circinata, Fusarium moniliforme, Alternaria alternata, Bipolaris sorghicola, Helminthosporium sorghicola, Curvularia lunata, Phoma insidiosa, Pseudomonas avenae (Pseudomonas alboprecipitans), Ramulispora sorghi, Ramulispora sorghicola, Phyllachara sacchari, Sporisorium reilianum (Sphacelotheca reiliana), Sphacelotheca cruenta, Sporisorium sorghi,* Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi, Rhizoctonia solani, Acremonium strictum, Sclerophthona macrospora, Peronosclerospora sorghi, Peronosclerospora philippinensis, Sclerospora graminicola, Fusarium graminearum, Fusarium oxysporum, Pythium arrhenomanes, Pythium graminicola,* etc.

Nematodes include parasitic nematodes such as root-knot, cyst, burrowing, reniform and lesion nematodes, including *Heterodera* and *Globodera spp;* particularly *Globodera rostochiensis* and *globodera pailida* (potato cyst nematodes); *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); and *Heterodera avenae* (cereal cyst nematode).

The methods of the invention can be used with other methods available in the art for enhancing disease resistance in plants.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

6-Methylsalicylate Induces PR Protein Accumulation in Plants

Maize (*Zea mays* inbred B73) plants were grown in Strong-Lite Universal Mix potting soil (Universal Mix, Pine Bluff, AZ) and raised in a greenhouse (16-h day, 20 to 35 C, 50% relative humidity, 560 to 620 μE s-1 m-2 of light from both sunlight and halogen lamps). To determine the effect of 6-methylsalicylic acid or salicylic acid on accumulation of defense proteins in maize, leaves of seedlings at the V-4 stage were infiltrated with an aqueous solution containing a buffer and either 6-methylsalicylate or salicylate at concentrations ranging from 0 to 2.0 mM. The leaf blade of the fifth leaf was infiltrated from the underside using a needle-less disposable syringe with a solution of the test compound in 1 mM sodium acetate buffer, pH 5.5. Three plants were used for each treatment. Seven days after infiltration, proteins were extracted from leaf tissue by acidic extraction. Leaf tissues were homogenized in 100 mM sodium phosphate-citrate buffer, pH 2.8, containing 6 mM ascorbic acid and 1% (*v/v*) 2-mercaptoethanol. The acidic extracts were mixed with one-fourth volume of 5X reducing buffer (312.5 mM Tris, 10% (*w/v*) sodium dodecyl sulfate, 50% (*v/v*) glycerol, 25% (*v/v*) 2-mercaptoethanol, 0.05% (*w/v*) bromophenol blue) and incubated at 100°C for 5 minutes immediately prior to being subjected to denaturing polyacrylamide gel electrophoresis on 4-20% Tris-glycine gradient gels obtained from Novex (San Diego, CA). The leaf proteins were transferred from the gel to a PVDF membrane (0.2 μm) and subjected to western blot analysis using antiserum raised against tobacco PR1a and chemiluminescent detection.

At all concentrations tested, both 6-methylsalicylate-treated leaves and salicylate-treated leaves had dramatically higher levels of PR1-like proteins than did either untreated leaves or buffer-infiltrated leaves (Figure 1). At the lowest concentration tested, 0.5 mM, salicylate-treated leaves had a higher level of PR1-like proteins than did 6-methylsalicylate-treated leaves. However, the 6-

methylsalicylate utilized in this and the following experiments was not pure and thus, the actual concentration of 6-methylsalicylate in the solutions was most likely lower than indicated in Figure 1.

A similar experiment was conducted with Xanthi-nc tobacco plants grown under conditions as described *supra* for maize. Leaf six of eight-week old plants was syringe-infiltrated with an aqueous solution of 5 mM Bis-tris, pH 6.5 alone or with the addition of either 2.5 mM 6-methylsalicylate or 2.5 mM salicylate. Proteins were acidic-buffer extracted from leaves seven days after infiltration and analyzed by western blotting using antisera raised against tobacco PR proteins as described *supra* for maize. Both 6-methylsalicylate-treated leaves and salicylate-treated leaves had dramatically higher levels of PR1, glucanase and chitanase than did either untreated leaves or buffer-infiltrated leaves (Figure 2).

Thus, the experiments with maize and tobacco reveal that 6-methylsalicylic acid, like salicylic acid, is able to induce the accumulation of PR proteins in both a monocot maize and a dicot, tobacco. Because the PR proteins are components of systemic acquired resistance in plants, these results suggest that 6-methylsalicylic acid is an inducer of disease resistance in plants.

EXAMPLE 2

Effects of Foliar Application of 6-Methylsalicylate on TMV-Induced Lesions in Tobacco

To test the ability of 6-methylsalicylate to induce disease resistance in plants, leaves of tobacco plants were treated as described in Example 2. Seven days after treatment, treated leaves (leaf 6) were inoculated with 0.1 mL of an aqueous suspension containing 5 μg tobacco mosaic virus (TMV) particles per mL. The area of 50 TMV-induced lesions on leaf 6 was measured on five plants for each treatment. Mean lesion area was significantly smaller on 6-methylsalicylate-treated leaves and salicylate-treated leaves as compared to either similar untreated or buffer-treated leaves. Mean lesion area for the control and buffer-treated leaves was about 1 and 1.2 mm^2, respectively. For the 6-methylsalicylate-treated leaves and salicylate-treated leaves, mean lesion areas were only about 0.65 and 0.35 mm^2, respectively. Although mean lesion area was smallest for the salicylate-

treated leaves, the results revealed that 6-methylsalicylate induces resistance in tobacco plants to disease caused by TMV.

EXAMPLE 3

Assessing the Antifungal activity of 6-Methylsalicylic Acid and its Analogues

To test the antifungal activity of 6-methylsalicylic acid and analogs, in vitro assays were conducted following the method described by Duvick, J.P. *et al.* ((1992) *J. Biol. Chem. 267*: 18814-18820). Compounds were applied to ungerminated spores. In addition to 6-methylsalicylic acid (6-MeSA), 3-methylsalicylic acid (3-MeSA), 4-methylsalicylic acid (4-MeSA) and 5-methylsalicylic acid (5-MeSA) were tested. All compounds were applied at a rate of 100 μg/mL. The fungi selected for the study were four maize pathogens, *Aspergillus flavus*, *Fusarium graminearum, Fusarium moniliforme* and *Cercospora zea-maydis*. Inhibition of spore germination and hyphal growth was assessed at 24 hours after the compounds were applied to the fungi. Antifungal activity was scored on a scale of zero to four with zero equal to no inhibition of spore germination and hyphal growth and four equal to total inhibition of spore germination and hyphal growth. Unlike its analogues, 6-methlysalicylic acid had no antifungal activity against any of the fungi tested at 100 μg/mL (Figure 3). Both 3-methylsalicylic acid and 4-methylsalicylic acid had strong antifungal activity against all the maize pathogens except *A. flavus*. For 5-methylsalicylic acid, intermediate antifungal activity was detected against *Fusarium graminearum* and *Cercospora zea-maydis*, and no activity was observed against *Fusarium moniliforme* and *Aspergillus flavus*.

The observation that 6-methlysalicylic acid had no antifungal activity against the four maize pathogens was unexpected because 6-methylsalicylic acid was previously reported to have antimicrobial activity against fungi and bacteria (Venkatasubbaiah *et al.* (1992) *Mycologia 84*: 715-723; Venkatasubbaiah *et al.*(1994) *Plant Dis. 79*:1157-1160).

A second experiment to evaluate the antifungal activity of 6-methylsalicylic acid and its analogs was conducted. Compounds were assayed at 0, 3.13, 6.25, 12.5, 25, 590, 100, and 200 μg/mL using the method described by Duvick, J.P. *et*

al. ((1992) *J. Biol. Chem. 267*: 18814-18820). The effects of the compounds on spore and hyphal growth of *Aspergillus flavus*, *Fusarium graminearum*, *Fusarium moniliforme*, *Colletotrichum graminicola* and *Cochliobolus heterostrophus* were investigated. Similar to the first experiment, 6-methylsalicylic acid displayed the lowest level of antifungal activity of the compounds tested (Tables 1 and 2). A concentration of 200 μg/mL 6-methylsalicylic acid or higher was required to achieve detectable inhibition of spore germination and hyphal growth for four of five fungal species tested (Table 1). *Colletotrichum graminicola* was more sensitive to 6-methylsalicylic acid than the other fungi. At a concentration of 100 μg/mL, 6-methylsalicylic acid displayed some antifungal activity against this fungus (Table 1). However, spore germination and hyphal growth of *Colletotrichum graminicola* could not be completed arrested at a concentration of 200 μg/mL (Table 2). All the other compounds tested achieved complete spore germination and growth inhibition of *Colletotrichum graminicola* at 100 μg/mL. Unlike its structural analogs, 6-methylsalicylic acid was not able to completely arrest germination and growth of any of the fungi tested at concentration of 200 μg/mL or lower.

Table 1

Minimum concentration resulting in detectable inhibition of spore germination and hyphal growth (μg/mL)					
Compound	*Aspergillus flavus*	*Colletotrichum graminicola*	*Cochliobolus heterostrophus*	*Fusarium graminearum*	*Fusarium moniliforme*
3-MeSA	100	100	100	100	200
4-MeSA	100	100	100	100	200
5-MeSA	200	50	3.13	200	200
6-MeSA	>200	100	>200	200	>200

Table 2

Minimum concentration resulting in complete inhibition of spore germination and hyphal growth (μg/mL)					
Compound	*Aspergillus flavus*	*Colletotrichum graminicola*	*Cochliobolus heterostrophus*	*Fusarium graminearum*	*Fusarium moniliforme*
3-MeSA	200	100	200	200	200
4-MeSA	200	100	200	200	200
5-MeSA	200	100	200	200	200
6-MeSA	>200	>200	>200	>200	>200

EXAMPLE 4

Genetically Engineering Plants to Produce 6-Methylsalicylic Acid.

Agrobacterium-mediated transformation of Xanthi-nc NN- and nn-genotype tobacco was accomplished using the 19.9 kb vector comprising: (1) an SCP1 promoter operably linked to a nucleotide sequence comprising a chloroplast-targeting sequence operably linked to a 6-methylsalicylic acid synthase coding sequence; and (2) an UCP3 promoter operably linked to a NPTII selectable marker gene. The chloroplast-targeting sequence (SEQ ID NO: 13) comprises from the 5'-end, nucleotides 45-150 of GenBank Accession No. M18320 fused to nucleotides 151-215 of the sequence in plasmid SLJ2524 (Scofield *et al.* (1994) *Mol. Gen. Genet. 244*, 189-196. The 6-methylsalicylic acid synthase coding sequence is from EMBL Accession No. X55776 (SEQ ID NO: 1).

Leaf samples were extracted as previously described for analysis of total salicylic acid (free salicylic acid + conjugated salicylic acid) (Enyedi *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:2480-2484). Twenty, independent primary transformants (T_0) were identified that produced 6-methylsalicylic acid. T_0 plants that produced 6-methylsalicylic acid were identified by the co-elution of plant-extracted, putative 6-methylsalicylic acid with authentic 6-methylsalicylic acid by HPLC using fluorescence and absorption detectors and by its absorption spectrum using a diode array detector. The identity of the plant-produced 6-methylsalicylic acid was confirmed by GLC-mass spectrometry using trimethylsilylated plant

extract. For a number of primary transformants, the levels of 6-methylsalicylic acid in leaves were estimated to exceed 10 μg/g fresh weight at time of flowering and with the levels continuing to increase as the plant matured. For one plant, the level of 6-methylsalicylic acid in its leaves exceeded 30 μg/g fresh weight. Because 6-methylsalicylic acid was not detected in unhydrolyzed tissue extracts, but was detected following hydrolysis, 6-methylsalicylic acid likely exists predominantly in a conjugated form *in planta*.

T_0 plants were selfed and allowed to produce seed. Following seed germination and subsequent seedling growth, leaves from the T_1 progeny of a T_0 plant (SID #870956), that was positive for 6-methylsalicylic acid accumulation in its leaves, were analyzed to determine the level of 6-methylsalicylic acid. Of the T_1 progeny analyzed, 11 of a total of 13 plants had detectable levels of 6-methylsalicylic acid. In leaves from untransformed control plants (Xanthi-nc NN-genotype), 6-methylsalicylic acid was not detected. The levels of 6-methylsalicylic acid in leaves of the 11 plants ranged from less than 1 μg/g fresh weight to about 9 μg/g fresh weight. These results reveal that transforming a plant with a 6-methylsalicylic acid synthase causes the accumulation of 6-methylsalicylic acid in the plant and that this trait is inherited by the progeny of the transformed plant.

The same leaves from the T_1 progeny of T_0 plant SID #870956 that were analyzed for 6-methylsalicylic acid levels were also subjected to western blot analyses utilizing antibodies raised against tobacco PR1 and antibodies raised against tobacco chitinase. Leaves from T_1 plants that had high levels of 6-methylsalicylic acid were also observed to have high levels of PR1 and chitinase proteins, relative to control leaves, suggesting the accumulation of such defense-related proteins is positively correlated with the accumulation of 6-methylsalicylic acid in a plant leaf. High levels of both PR1 and chitinase were also detected in untransformed tobacco leaves that were inoculated with TMV four days before the leaves were harvested.

To quantify the PR1 and chitinase protein levels, the western blots were scanned, and the PR1 and chitinase bands on the images were analyzed using image analysis software (Version 1.5 of 'NIH Image' for Macintosh, National Institutes of Health, USA) (Figure 4). The intensity of PR1 and chitinase bands for

the transgenic plants are expressed as a percentage of the band intensity of the corresponding protein bands detected in a protein extract from a tobacco mosaic virus (TMV)-inoculated untransformed control plant. The results in Figure 4 reveal that the levels of the PR1 and chitnase proteins in the leaves of the trangenic tobacco plants are positively corrrelated with the level of 6-methylsalicylic acid present in the same leaves.

The incorporation of the 6-methylsalicylic acid synthase gene does not appear to have a detrimental effect on plant phenotype. All T_0 plants had a normal morphology and produced viable seed. T_1 plants produced from SID #870956 showed variability in height and branching pattern. However, this variation is not likely to be the result of any adverse effects of the accumulation of 6-methylsalicylic acid in the plants because the levels of 6-methylsalicylic acid detected in the leaves did not correlate with the phenotypic variability observed.

EXAMPLE 5

Effect of the Level of 6-Methylsalicylate Acid on Resistance against Tobacco Mosaic Virus in Transgenic Tobacco Plants Expressing a 6-Methylsalicylic Acid Synthase Gene

Two independent T_0, NN *xanthi* NC tobacco plants (SID# 911403 and 870955) transformed with the 6-methylsalicylic acid synthase gene construct described in Example 4 were selfed. T_1 progeny possessing the 6-methylsalicylic acid synthase gene were selected by germinating seeds on a medium containing kanamycin. After three weeks, the largest of germinated seedlings were transferred into potting mix and raised in a greenhouse. The youngest, nearly fully expanded leaf from plants that were at about the six-leaf stage was harvested from each plant and analyzed for 6-methylsalicylate content and PR protein levels. One week later the youngest, nearly fully expanded leaf from these plants was inoculated with tobacco mosaic virus (TMV). The leaf was lightly dusted with carborundum powder and inoculated with 0.2 mL of an aqueous suspension containing 5 μg tobacco mosaic virus (TMV) particles per mL. The area of 40 TMV-induced lesions on each inoculated leaf was measured four days later. Mean lesion area showed an inverse correlation with tissue 6-methylsalicylate levels

(Figure 5). The results indicate that increasing the level of 6-methylsalicylic acid in a plant increases disease resistance in the plant.

EXAMPLE 6

Transformation and Regeneration of Transgenic Maize Plants by Particle Bombardment

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a DNA construct comprising a chloroplast transit peptide operably linked to the nucleotide sequence encoding the 6-methylsalicylic acid synthase from *Penicillium patulum*. The DNA construct additionally comprises an ubiquitin promoter operably linked to the sequence encoding the 6-methylsalicylic acid synthase. The plasmid also contains the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37) that confers resistance to the herbicide Bialaphos. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

This plasmid is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 μL prepared tungsten particles in water
10 μL (1 μg) DNA in TrisEDTA buffer (1 μg total)
100 μL 2.5 M $CaCl_2$
10 μL 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 mL 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µL 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µL spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 ml/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 120.0 g/L sucrose, 1.0 mg/L 2,4-D, and 2.88 g/L L-proline (brought to volume with D-I H20 following adjustment to pH 5.8 with KOH); 2.0 g/L Gelrite (added after bringing to volume with D-I H20); and 8.5 mg/L silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 ml/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 30.0 g/L sucrose, and 2.0 mg/L 2,4-D (brought to volume with D-I H20 following adjustment to pH 5.8 with KOH); 3.0 g/L Gelrite (added after bringing to volume with D-I H20); and 0.85 mg/L silver nitrate and 3.0 mg/L bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 ml/L MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H20) (Murashige and Skoog (1962) Physiol. Plant. 15:473), 100 mg/L myo-inositol, 0.5 mg/L zeatin, 60 g/L sucrose, and 1.0 ml/L of 0.1 mM

abscisic acid (brought to volume with polished D-I H20 after adjusting to pH 5.6); 3.0 g/L Gelrite (added after bringing to volume with D-I H20); and 1.0 mg/L indoleacetic acid and 3.0 mg/L bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 ml/L MS vitamins stock solution (0.100 g/L nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H20), 0.1 g/L myo-inositol, and 40.0 g/L sucrose (brought to volume with polished D-I H20 after adjusting pH to 5.6); and 6 g/L bacto-agar (added after bringing to volume with polished D-I H20), sterilized and cooled to 60° C.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for 6-methylsalicylic acid content.

EXAMPLE 7

Maize Plants Transformed with the 6-Methylsalicylic Acid Synthase Gene from *Penicillium patulum*

To produce transgenic maize plants that express the *Penicillium patulum* 6-methylsalicylic acid synthase gene, GS3 maize embryos were transformed by particle bombardment with a plasmid comprising: (1) a ubiquitin promoter

operably linked to a nucleotide sequence comprising a chloroplast-targeting sequence operably linked to a 6-methylsalicylic acid synthase coding sequence from EMBL Accession No. X55776 (SEQ ID NO: 1); and (2) an actin promoter operably linked to a monocot-optimized PAT selectable marker gene.

The chloroplast-targeting sequence is set forth in SEQ ID NO: 14. This sequence was prepared by optimizing for expression, the chloroplast targeting sequence, particularly nucleotides 1013-1168, of EMBL Accession No. X53398. The optimized chloroplast-targeting sequence (SEQ ID NO: 14) is a novel synthetic sequence in which base substitutions were made in the sequence of EMBL Accession No. X53398 to alter or eliminate certain structural features that can reduce expression, particularly hairpins and dimers. The polypeptide chain encoded by SEQ ID NO: 14 is, however, identical to that encoded by the corresponding region of EMBL Accession No. X53398.

From 103 callus lines that were RT-PCR positive for 6-methylsalicylic acid synthase expression, 89 T_0 plant events were generated, of which 60 events were RT-PCR positive for 6-methylsalicylic acid synthase expression. All T_0 plants from these 60 RT-PCR positive events along with plants from six RT-PCR negative lines (control lines) were analyzed for 6-methylsalicylic acid accumulation. A total of 402 plants were sampled. By HPLC analysis 25 events (total 84 plants) were identified that had detectable 6-methylsalicylic acid accumulation. The levels of 6-methylsalicylic acid ranged from 0.1 to 11.8 μg/g fresh weight and were thus on a similar order of magnitude as what had been observed in transgenic 6-methylsalicylic acid synthase-tobacco with enhanced resistance to tobacco mosaic virus described in Example 5. The levels of 6-methylsalicylic acid increased in the maize plants transformed with the 6-methylsalicylic acid synthase gene as the plants matured (Figure 6). A similar maturation effect was also observed with the tobacco plants transformed with the 6-methylsalicylic acid synthase gene described in Example 4. Some transgenic maize events had 6-methylsalicylic acid accumulation associated with a patchy necrosis leaf phenotype but formed normal ears. However, the plant that had the highest levels of 6-methylsalicylic acid in its leaves had a normal (wild-type) phenotype. Seed from the T_0 plants was harvested.

The level of 6-methylsalicylic acid and salicylic acid in kernels (T_1) from T_0 transgenic maize was assessed. For each plant tested, ten kernels from the middle of the dried-down ear of a T_0 GS3 maize plant that had been pollinated with HG11 pollen were bulked and extracted in triplicate. Levels of 6-methylsalicylic acid and salicylic acid in hydrolyzed extracts was determined using HPLC as described in Example 4. Kernels from seed of nontransgenic GS3 plants that were pollinated with HG11 were used as controls. The results from four transformed plants are presented in Figure 7. Both 6-methylsalicylic acid and salicylic acid was detected in all transformed plants. The average levels of 6-methylsalicylic acid in kernels from the transformed plants ranged from less than 0.5 μg/g dry kernel to about 4.5 μg/g dry kernel. Salicylic acid, but not 6-methylsalicylic acid, was detected in kernels from the control plants. For all plants examined, average kernel salicylic acid levels did not exceed 1 μg/g dry kernel.

It should be noted that not all the maize kernels used to determine 6-methylsalicylic acid levels are expected to possess a copy of the 6-methylsalicylic acid synthase gene and thus would not be expected to produce 6-methylsalicylic acid. For example, if a T_0 parent plant contained a single copy of the 6-methylsalicylic acid gene in its genome and was cross pollinated with HG11 pollen, (HG11 does not possess a 6-methylsalicylic acid gene in its genome), the resulting ear is expected to have a 1:1 ratio of kernels with the 6-methylsalicylic acid gene to kernels lacking the gene. Assuming, for example, that 6-methylsalicylic acid detected in a kernel is produced in the kernel and not produced in maternal tissues and translocated to the developing kernel, an estimate of 6-methylsalicylate acid from a group of ten kernels resulting from the such a cross would be expected to underestimate 6-methylsalicylic acid levels in kernels possessing the gene by about 50%. While it is not known whether 6-methylsalicylic acid is translocated in a plant, the methods of the present invention do not depend on such translocation, only that transforming a plant with a 6-methylsalicylic acid synthase gene can increase the level of 6-methylsalicylic acid in the plant.

The levels of 6-methylsalicylic acid were determined in the leaves from T_1 plants that were grown from the T_1 kernels described above (Figure 8). Only plants that were positive for 6-methylsalicylic acid synthase gene expression as

determined by RT-PCR were included in the analyses. The levels of 6-methylsalicylic acid ranged from about 0 to nearly 40 μg/g dry weight leaf tissue. Thus, the results disclosed herein demonstrate that the traits of 6-methylsalicylic acid synthase gene expression and the accumulation of 6-methylsalicylic acid can be inherited from one generation to the next.

EXAMPLE 8

Agrobacterium-Mediated Transformation and Regeneration of Transgenic Maize Plants

For *Agrobacterium*-mediated transformation of maize with a polyketide synthase gene or other nucleotide sequence of the invention, preferably the method of Zhao *et al.* is employed (U.S. Patent No. 5,981,840), the contents of which are hereby incorporated by reference. Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the polyketide synthase gene or other nucleotide sequence of interest to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into

plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

A transformation vector was constructed for *Agrobacterium*-mediated maize transformation with the 6-methylsalicylic acid synthase gene. The vector comprises between the right and left borders: (1) a ubiquitin promoter operably linked to a nucleotide sequence comprising a chloroplast-targeting sequence operably linked to a 6-methylsalicylic acid synthase coding sequence from EMBL Accession No. X55776 (SEQ ID NO: 1); and (2) an actin promoter operably linked to a monocot-optimized PAT selectable marker gene. The chloroplast targeting sequence is the optimized chloroplast targeting sequence (SEQ ID NO: 14) described in Example 7 *supra*.

Maize transformation with this vector was initiated. Two hundred and twenty-six single plant events that are RT-PCR positive for 6-methylsalicylate synthase expression have been produced.

EXAMPLE 9

Canola Plants Transformed with a 6-Methylsalicylic Acid Synthase Gene Produce 6-Methylsalicylic Acid

Canola (*Brassica* sp.) plants were transformed with one of three vectors. Each vector comprises an SCP1 promoter operably linked to a nucleotide sequence comprising a 6-methylsalicylic acid synthase coding sequence (EMBL Accession No. X55776, SEQ ID NO: 1) and an operably linked chloroplast-targeting sequence. See, for the SCP1 promoter, WO 97/47756 and WO 99/43838; herein incorporated by reference. The chloroplast-targeting sequence (SEQ ID NO: 13) is described in Example 4. Vector 1 additionally comprises a PAT selectable marker gene operably linked to the 35S promoter. Vector 2 additionally comprises a PAT selectable marker gene operably linked to the SCP1 promoter. Vector 3 additionally comprises the nptII selectable marker gene operably linked to the UCP3 promoter (WO 97/47756; WO 99/43838).

The levels of 6-methylsalicylic acid were measured in leaves from regenerated transformed canola plants (T_0) and from an untransformed control plant as described for tobacco leaves in Example 4. Of nine transformed plants,

leaves from six plants had detectable levels of 6-methylsalicylic acid. In leaves from the control plant, 6-methylsalicylic acid was not detected. The levels of 6-methylsalicylic acid in the leaves of the transformed plants ranged from 0 to about 0.14 μg/g fresh weight. The highest level of 6-methylsalicylic acid was measured in leaves from a canola plant transformed with vector 2.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

THAT WHICH IS CLAIMED:

1. A method for enhancing disease resistance in a plant comprising stably incorporating in the genome of said plant a DNA construct comprising a nucleotide sequence encoding a polyketide synthase operably linked to a promoter capable of driving gene expression in a plant, wherein the level of at least one defense-related protein is increased in said plant.

2. The method of claim 1 wherein said defense-related protein is a pathogenesis-related protein.

3. The method of claim 1 wherein said polyketide synthase catalyzes the synthesis of a polyketide which is capable of inducing the synthesis of at least one defense-related protein in a plant.

4. The method of claim 1 wherein said polyketide synthase is a type I polyketide synthase.

5. The method of claim 4 wherein said type I polyketide synthase is a 6-methylsalicylic acid synthase.

6. The method of claim 5 wherein said 6-methylsalicylic acid synthase is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1-3.

7. The method of claim 1 wherein the level of 6-methylsalicylic acid is increased in said plant.

8. The method of claim 1 wherein said DNA construct further comprises an operably linked chloroplast-targeting sequence.

9. The method of claim 1 further comprising stably incorporating in the genome of said plant at least one additional DNA construct comprising a

nucleotide sequence encoding a protein that is capable of altering the level of a polyketide in a plant, said sequence operably linked to a promoter capable of driving gene expression in a plant.

10. The method of claim 9 wherein said additional DNA construct further comprises an operably linked chloroplast-targeting sequence.

11. The method of claim 9 wherein said protein is a phosphopantetheinyl transferase or a glucosyltransferase.

12. The method of claim 11 wherein said phosphopantetheinyl transferase is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 4-9.

13. The method of claim 11 wherein said glucosyltransferase catalyzes the transfer of at least one glucosyl residue to 6-methylsalicylic acid.

14. The method of claim 11 wherein said glucosyltransferase is encoded by a nucleotide sequence selected from the group consisting SEQ ID NOs: 10-12.

15. The method of claim 9 wherein said nucleotide sequence is in the antisense orientation.

16. A plant genetically manipulated for enhanced disease resistance comprising in its genome at least one stably incorporated DNA construct comprising a nucleotide sequence encoding a polyketide synthase operably linked to a promoter capable of driving gene expression in a plant, wherein the level of at least one defense-related protein is increased in said plant.

17. The plant of claim 16 wherein said polyketide synthase catalyzes the synthesis of a polyketide which is capable of inducing the synthesis of at least one defense-related protein in a plant.

18. The plant of claim 16 wherein said polyketide synthase is a type I polyketide synthase.

19. The plant of claim 18 wherein said type I polyketide synthase is 6-methylsalicylic acid synthase.

20. The plant of claim 16 wherein said DNA construct further comprises an operably linked chloroplast-targeting sequence.

21. The plant of claim 16 further comprising in its genome at least one stably incorporated additional DNA construct comprising a nucleotide sequence encoding a protein that is capable of altering the level of a polyketide in a plant, said sequence operably linked to a promoter capable of driving gene expression in a plant.

22. The plant of claim 21 wherein said additional DNA construct further comprises an operably linked chloroplast-targeting sequence.

23. The plant of claim 21 wherein said protein is a phosphopantetheinyl transferase or a glucosyltransferase.

24. The plant of claim 23 wherein said glucosyltransferase catalyzes the transfer of at least one glucosyl residue to 6-methylsalicylic acid.

25. The plant of claim 21 wherein said nucleotide sequence is in the antisense orientation.

26. The plant of claim 16 wherein said plant is a monocot.

27. The plant of claim 26 wherein said monocot is selected from the group consisting of maize, wheat, rice, barley, sorghum, oats and rye.

28. The plant of claim 16 wherein said plant is a dicot.

29. The plant of claim 28 wherein said dicot is selected from the group consisting of soybean, *Brassica* sp., sunflower, safflower, alfalfa, potato, peanut and cotton.

30. Seed of the plant according to any one of claims 16 to 29.

31. A plant cell genetically manipulated for enhanced disease resistance comprising in its genome at least one stably incorporated DNA construct comprising a nucleotide sequence encoding a polyketide synthase operably linked to a promoter capable of driving gene expression in a plant cell, wherein the level of at least one defense-related protein is increased in said plant cell.

Figure 1

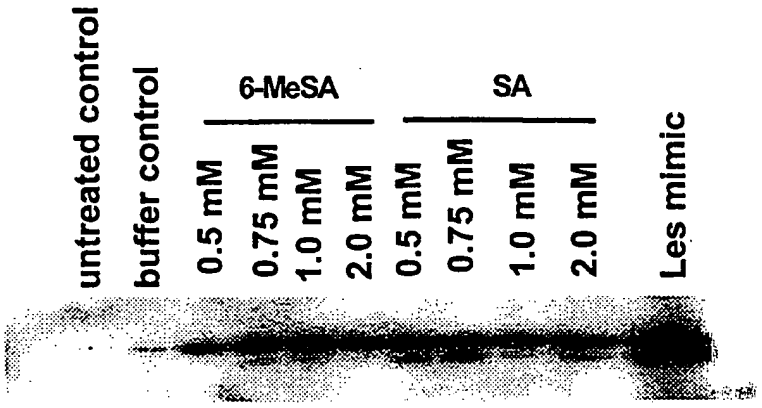

untreated control
buffer control
6-MeSA
SA
0.5 mM
0.75 mM
1.0 mM
2.0 mM
0.5 mM
0.75 mM
1.0 mM
2.0 mM
Les mimic

Figure 2

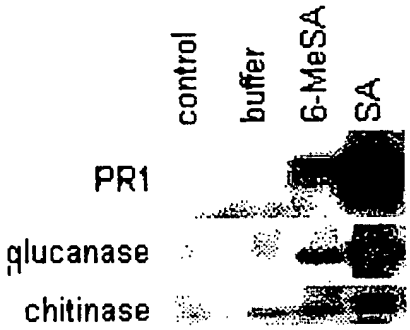


Figure 3

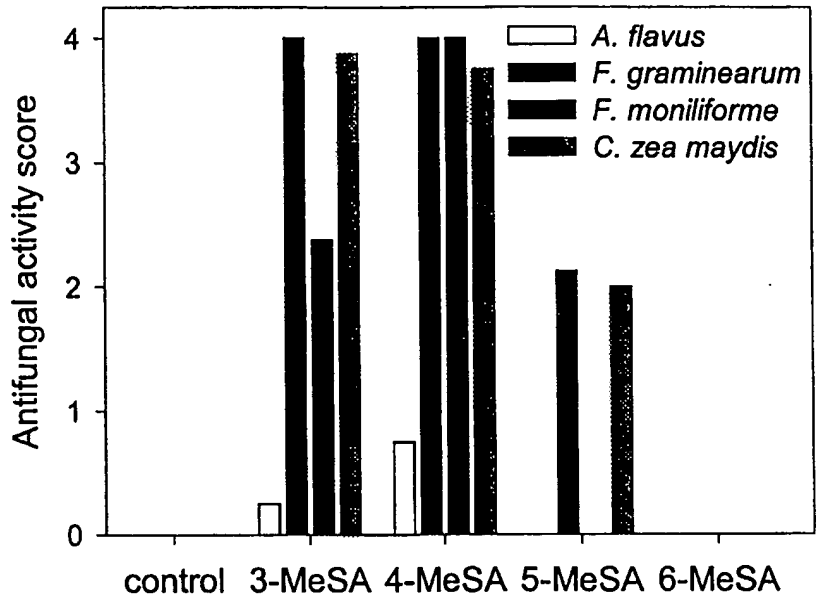

Antifungal activity score
0
1
2
3
4
A. flavus
F. graminearum
F. moniliforme
C. zea maydis
control
3-MeSA
4-MeSA
5-MeSA
6-MeSA

Figure 4

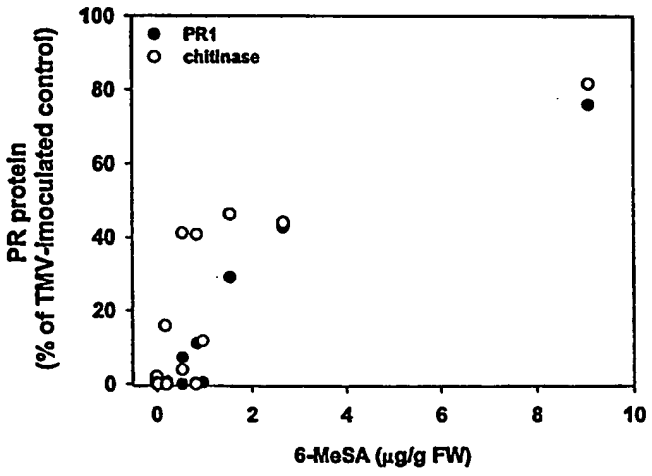
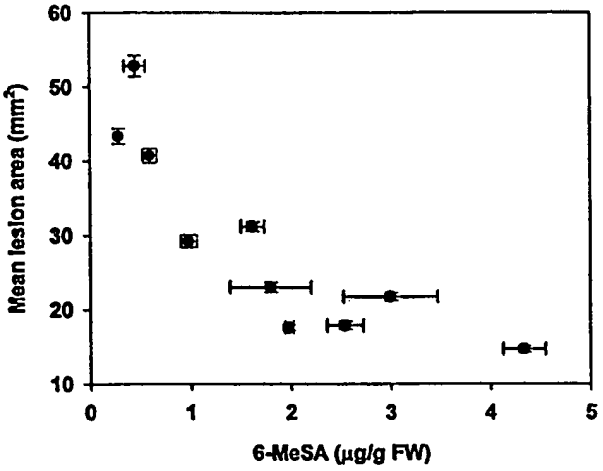
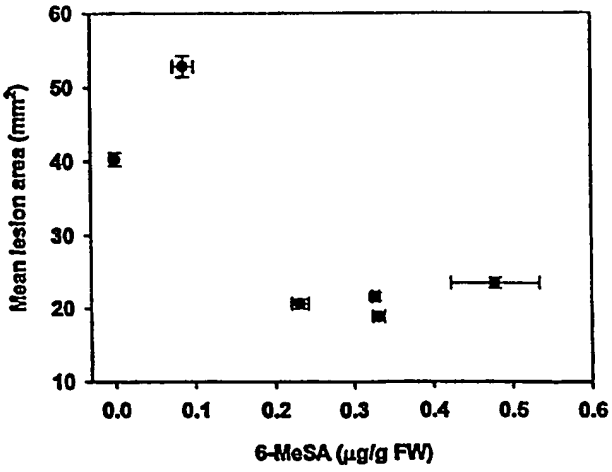

PR1
chitinase
PR protein
(% of TMV-imoculated control)
100
80
60
40
20
0
0
2
4
6
8
10
6-MeSA (µg/g FW)

Figure 5

A


Mean lesion area (mm²)
60
50
40
30
20
10
0
1
2
3
4
5
6-MeSA (µg/g FW)

B


Mean lesion area (mm²)
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50
40
30
20
10
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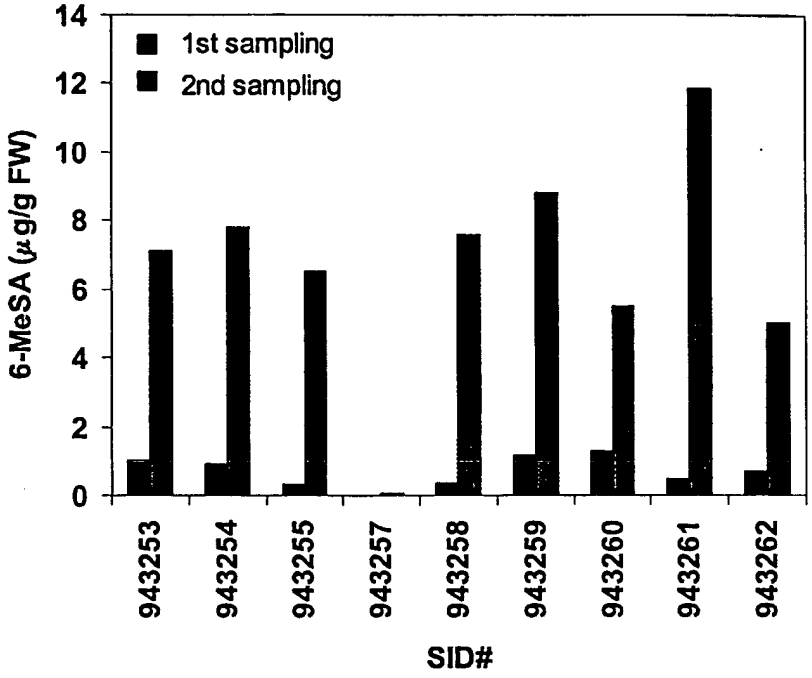
0.1
0.2
0.3
0.4
0.5
0.6
6-MeSA (µg/g FW)

Figure 6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12
10
8
6
4
2
0
1st sampling
2nd sampling
6-MeSA (µg/g FW)
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943255
943257
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943259
943260
943261
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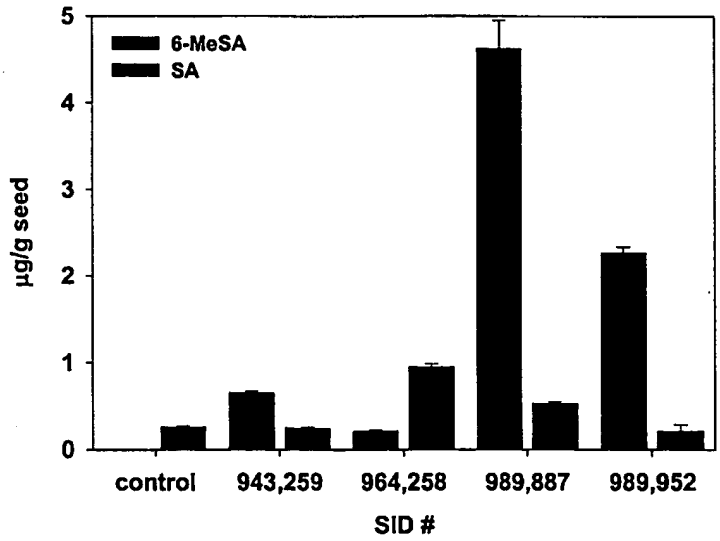
SID#

Figure 7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µg/g seed
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2
3
4
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control
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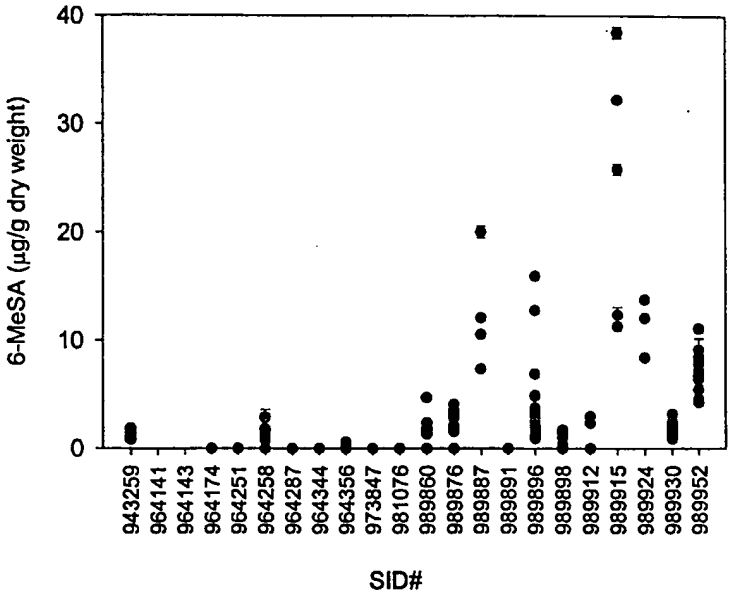
964,258
989,887
989,952
SID #

Figure 8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0
6-MeSA (µg/g dry weight)
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SEQUENCE LISTING

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<120> METHODS FOR ENHANCING THE DISEASE RESISTANCE OF PLANTS

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<220>
<223> Description of Artificial Sequence: sequence
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INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 00/04691

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H1/00 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 02669 A (KOSAN BIOSCIENCES INC) 21 January 1999 (1999-01-21)	1-12, 15-23, 25-31
Y	page 4, line 25 -page 5, line 28 page 7, line 28 -page 8, line 3 page 9, line 21 -page 11, line 18 page 13, line 9 - line 13 page 16, line 1 - line 8 page 23, line 29 - line 32; examples 1,5-7	13,14,24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2000	30/06/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter... nal Application No
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